(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 687 731 A1**

# (12) EUROPEAN PATENT APPLICATION

(21) Application number: 95202092.3

(22) Date of filing: 08.11.91

(51) Int. Cl.[6]: C12N 15/12, C12N 15/62, C12N 15/70, C12N 1/21, C07K 14/815, //(C12N1/21, C12R1:19)

This application was filed on 01 - 08 - 1995 as a divisional application to the application mentioned under INID code 60.

(30) Priority: 08.11.90 JP 303096/90
13.02.91 JP 41271/91

(43) Date of publication of application:
20.12.95 Bulletin 95/51

(60) Publication number of the earlier application in accordance with Art.76 EPC: 0 511 393

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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## (54) Secretion vector, transformed microorganisms containing said vector and manufacture of products from said microorganism

(57) A secretion vector for foreign protein production, in particular hirudin HV1, hirudin HV3 and hirudin analog HV1C3, is provided comprising a replication origin (ori) of a pUC plasmid, a tac or a trp promoter, a DNA sequence encoding signal peptide, and a DNA sequence encoding a foreign protein, all linked in an operable manner. Also provided are a method of producing said hirudins and hirudin analog employing the secretion vectors, and transformed microorganisms such as E. coli.

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## Technical Field

The present invention relates to secretion vectors for foreign protein production, transformed microorgamsms containing said vectors, and a method of producing hirudin or its analogs by using transformed microorganisms.

The secretion vectors for secretion of foreign proteins such as the hirudin analog described in the present invention facilitates the industrially advantageous production of foreign proteins such as hirudin analogs, since they enable high yield extracellular production of foreign proteins when E. coli is transformed with said vectors.

## Background Art

Hirudin is an anti-coagulation factor secreted from the salivary glands of medicinal leeches, Hirudo medicinalis, and is a mixture of peptides consisting of 65 and 66 amino acids. The structure of hirudin was determined by Dodt et al. [FEBS Lett. 165, 180(1984)] as hirudin variant 1 (HV1). Another variant, hirudin HV2, [Harvey et al., Proc.Natl.Acad. Sci. USA, 83, 1084(1986)] has nine different amino acids in comparison with hirudin HV1, and still another variant, hirudin HV3, [Dodt et al., Biol. Chem. Hoppe-Seyler, 367, 803-(1986)] has ten different amino acids in comparison with HV2, having the same sequence as HV2 up to $Ser^{32}$, and inserting an amino acid ($Ala^{63}$) in the C-terminal region. The structures of these three hirudin variants are shown in Fig. 1.

These natural type variants comprise 65 or 66 amino acids, and two domains are discernible. These are the spherical structured N-terminal region with three disulfide bonds, and the acidic C-terminal region exhibiting homology with the thrombin cleaving region of the prothrombin molecule, or with the fibrinogen cleaving region.

The present inventors have discovered that HV1 contains $Leu^{64}$-$Gln^{65}$, while HV3 contains $Asp^{65}$-$Glu^{66}$ in the C-terminal amino acid region. A patent application has been filed (Japanese Laid Open Patent Publication 3-164184 (1991)) on the synthesis of synthetic genes for HV1 and HV3 and their expression in E. coli.

Hirudin variants HV1, HV2 and HV3, having anti-thrombotic activity, are not acceptable drugs for clinical use because of their serious side effects, such as prolongation of bleeding time.

Several systems for producing hirudin by genetic engineering technology have been proposed; however, a satisfactory method has not yet been developed. From an industrial point of view, an extracellular production system is particularly desirable, since if the produced protein can be secreted extracellularly, there will be advantages not only simplifying the separation and purification of the product because of its presence in active form, but also because the product will be protected from digestion by intracellular bacterial proteases.

Methods using Bacillus subtilis, yeast or E. coli as hosts have been proposed for the production of hirudin by secretion.

Methods using Bacillus subtilis as host are disadvantageous in that plasmids are generally unstable in this bacterium, resulting in frequent curing of plasmids, making the stable protein production difficult, or the protein products in the medium are likely to be digested by its own proteases also secreted into the medium. Methods proposed for hirudin production (for example Japanese Laid Open Patent Publication 2-35084 (1990)) have not solved such problems, and the production yield is only about 100 mg / L.

In methods using yeast as hosts, it is known that the C-terminal amino acids of the products are hydrolyzed by carboxypeptidase.

In a prior report (N. Riehl-Bellon et al., Biochemistry 1989, 28, 2941-2949), by-products having 1 or 2 amino acid residues hydrolyzed off from the C-terminal end of HV2 are disclosed.

To overcome this problem, a method using carboxypeptidase-deficient yeasts (Japanese Laid Open Patent Publication 2-104279 (1990)) as hosts has been proposed, but has not led to sufficient productivity.

Using E. coli as host, a method using the alkaline phosphatase signal sequence has been reported (J. Dodt et al., FEBS Lett. 202, 373-377 (1986)). Although this is a secretion system, the product is secreted mainly into the periplasmic space, and is not satisfactory since it requires the disruption of bacterial cells by an additional recovery process step such as osmotic shock, and the product yield is as low as 4 mg / l.

## Disclosure of Invention

The present inventors have produced various hirudin analogs based on the primary structure of the aforesaid hirudin HV1, HV2 and HV3 to compare their properties in animal models. They discovered out

that a hirudin analog (chimeric hirudin) having the hirudin HV1 amino acid sequence up to the 52nd amino acid and thereafter substituted with the sequence of HV3 exhibited not only high anti-thrombin activity, but also shortening of the prolongation of bleeding time.

Moreover, the present inventors have actively studied the extracellular production of foreign proteins in high yield using E. coli as host, and completed the present invention by discovering that when a replication origin (ori) of a pUC plasmid is used as a replication origin in a secretion vector with the DNA sequence for a signal peptide and a promoter, foreign protein can be secreted from E. coli in large quantity.

Therefore, the present invention relates to a new hirudin analog having high anti-thrombin activity.

Also, the present invention relates to a DNA sequence encoding the amino acid sequence of the hirudin analog having the aforesaid high anti-thrombin activity and low bleeding tendency, transformed microorganisms produced by transforming E. coli with expression vectors including said DNA sequence, and a method for manufacturing hirudin analogs by expressing said DNA sequence using the aforesaid transformed microorganisms, and recovering said hirudin analog.

The present invention also relates to an anti-coagulant drug having the aforesaid hirudin analog as an active ingredient.

The new hirudin analog HV1C3 of the present invention has the amino acid sequence shown in the following primary structure:

```
Val Val Tyr Thr Asp Cys Thr Glu Ser Gly
1                   5                  10
Gln Asn Leu Cys Leu Cys Glu Gly Ser Asn
                   15                  20
Val Cys Gly Gln Gly Asn Lys Cys Ile Leu

                   25                  30
Gly Ser Asp Gly Glu Lys Asn Gln Cys Val
                   35                  40
Thr Gly Glu Gly Thr Pro Lys Pro Gln Ser
                   45                  50
His Asn Gln Gly Asp Phe Glu Pro Ile Pro
                   55                  60
Glu Asp Ala Tyr Asp Glu
                   65
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(Formula-1)

An exemplary DNA sequence encoding the amino acid sequence of this analog can be shown by the following formula:

```
GTT GTA TAC ACT GAT TGT ACT GAA TCT GGC  30

CAG AAC CTG TGT CTG TGT GAA GGA TCC AAC  60

GTT TGT GGT CAG GGT AAC AAA TGT ATC CTC  90

GGG TCT GAT GGT GAA AAG AAC CAG TGT GTT 120

ACT GGT GAA GGT ACC CCG AAA CCG CAG TCT 150

CAT AAC CAG GGT GAT TTC GAA CCG ATC CCG 180

GAA GAC GCG TAC GAT GAA
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The hirudin analog of the present invention shown in Formula -1 can be synthesized by chemical synthesis, or it can be produced by genetic engineering methodology.

To manufacture the hirudin analog by genetic engineering, first, as described in a later example, the hirudin HV1 secreting plasmids pMTSHV1 and pMKSHV1 were constructed and used to transform E. coli. Hirudin HV1 was produced by secretion using the transformed microorganisms. Plasmid pMTSHV1 comprises a promoter (Ptrp), a DNA sequence encoding a signal peptide (PhoA signal), a DNA sequence encoding the amino acid sequence of hirudin HV1, a replication origin (ori) and a DNA sequence including a translation termination signal (rrnBT$_1$T$_2$), as shown in Fig. 3. In the present invention, in order to substitute after the 52nd amino acid of hirudin HV1 with the amino acid sequence of HV3, the HV1 secreting plasmid pMTSHV1 was cleaved with a restriction enzyme to remove the DNA sequence encoding the amino acid sequence after the 52nd amino acid of hirudin HV1. On the other hand, the DNA sequence encoding the amino acid sequence beginning with the 53rd amino acid of the HV3 expression plasmid pUCHV3 was excised using a restriction enzyme. The DNA having the sequence encoding up to the 52nd amino acid of hirudin HV1 from plasmid pMTSHV1 and the DNA encoding the amino acid sequence beginning with the 53rd amino acid of hirudin HV3 derived from plasmid pUCHV3 were ligated with DNA ligase to construct the plasmid pMTSHV1C3 containing the DNA encoding the amino acid sequence of the hirudin analog of the present invention.

Plasmid pMTSHV1C3 comprises a DNA sequence including a promoter, signal peptide and replication origin (ori) derived from plasmid pMTSHV1, a DNA sequence encoding the 1st - 52nd amino acids of hirudin HV1 and a translation termination signal, and a DNA sequence encoding the amino acids beginning with the 53rd amino acid of hirudin HV3 derived from plasmid pUCHV3, which is inserted between the aforesaid DNA sequence coding the 1st - 52nd amino acids of hirudin HV1 and the translation termination signal.

The hirudin analog of the present invention is produced intracellularly and is secreted into the medium when plasmid pMTSHV1C3 is introduced into E. coli, followed by cultivation of the transformed microorganisms.

In the present invention, the hirudin analog was separated by generally known methods, and purified by methods such as column chromatography, reverse-phase HPLC, etc.

The obtained hirudin analog exhibited higher anti-thrombin activity and lower bleeding tendency compared with hirudin HV1 in the animal model. It can be formulated into an excellent anti-coagulant drug by preparing it by generally known methods of formulation. In other words, the hirudin analog of the present invention can be further prepared by any common methods using any common carriers for pharmaceutical use or additives for formulation. The pharmaceutical composition administered intravenously, intracutaneously, subcutaneously or intramuscularly, or non-orally topically. Although the proper dose will be determined for each case by considering factors such as the symptoms, age and sex of patient, it is generally in the range of 0.1 mg to 100 mg for an adult per day, and the total amount will be administered in one to several doses.

Moreover, as previously described, the present invention is also related to secretion vectors for foreign protein production, particularly to the secretion vector for high yield extracellular production of hirudin or its analog.

Also, the present invention is related to secretion vectors containing DNA sequences encoding hirudin or its analogs, microorganisms (E. coli) transformed with said secretion vectors, and methods of manufacturing hirudin or its analogs by culturing said transformed microorganisms and recovering the product from the medium.

The foreign protein secretion vector of the present invention comprises a replication origin (ori) derived from a pUC plasmid, a tac promoter or trp promoter, a signal peptide sequence and a DNA sequence encoding foreign protein.

The DNA fragment containing the replication origin (ori) of a pUC plasmid in the present invention can be prepared by cleaving commercially available pUC family plasmids, for example, pUC9, pUC18 or pUC19, with combinations of suitable restriction enzymes. For example, a DNA fragment of about 1440 base pairs, including a replication origin (ori) prepared by cleaving pUC18 with restriction enzymes Pvu I, or Pvu I and Pvu II, can be used as a pUC replication origin in the plasmids of the present invention.

The tac promoter and trp promoter of the present invention have the nucleotide sequences shown in Fig. 7 and 8, respectively, and can be easily synthesized by a DNA synthesizer, etc. Therefore, these promoters can be synthesized and ligated with fragments containing the replication origin (ori) of plasmid pUC18. Alternatively, they can be relatively easily prepared by ligating DNA fragments generated by cleaving commercially available plasmids with restriction enzymes. For example, plasmid pMK2 can be prepared using T4 DNA ligase by ligating DNA fragments including the DNA sequence of the tac promoter obtained by cleaving the commercially available plasmid pKK223-3 (Pharmacia) with restriction enzymes Pvu I and Nru I and a DNA fragment including a replication origin (ori) obtained by cleaving the commercially available plasmid pUC18 with restriction enzymes Pvu I and Pvu II.

Plasmid pMT1 can be prepared by removing the fragment including the DNA sequence of the tac promoter by cleaving the abovementioned plasmid pMK2 with restriction enzymes EcoRI and Eco47III, and then inserting a fragment including the trp promoter sequence synthesized by a DNA synthesizer.

For the DNA sequence of the signal peptides of the present invention, DNA sequences coding signal peptides of proteins localizing in the periplasm of E. coli can be used, for example enzymes such as alkaline phosphatase (pho A) and b-lactamase (bla), or outer-membrane proteins such as OmpA, OmpB and OmpF. These DNA fragments can easily be prepared using a DNA synthesizer.

Although any protein can be the foreign protein encompassed by the present invention, hirudin and its variants are particularly suitable. The amino acid sequences of these proteins are shown in Fig.1 as hirudins HV1, HV2, HV3 and HV1C3.

## Brief Description of the Drawings

- Fig. 1 shows the amino acid sequence of hirudins HV1, HV2, HV3, and HV1C3.
- Fig. 2 shows the DNA sequence of the phoA signal peptide.
- Fig. 3 shows an outline of the construction of hirudin HV1 secreting plasmid pMTSHV1.
- Fig. 4 shows an outline of the construction of plasmid pMKSHV1.
- Fig. 5 shows an outline of the construction of hirudin HV1C3 secreting plasmid pMTSHV1C3.
- Fig. 6 (A) shows the C4 reverse phase HPLC profile of hirudin HV1; (B) shows the C4 reverse phase HPLC profile of hirudin HV1C3, respectively.
- Fig. 7 shows the DNA sequence of the tac promoter used in the present invention.
- Fig. 8 shows the DNA sequence of the trp promoter used in the present invention.
- Fig. 9 shows an outline of the construction of hirudin HV3 secreting plasmid pMTSHV3.
- Fig. 10 shows the DNA sequence of the signal peptide PhoA for hirudin HV3 secretion used in the example 5.
- Fig. 11 shows the profile of purified hirudin HV3 by C4 reverse phase HPLC.

## Best Mode for Carrying Out the Invention

The procedures for constructing plasmids pUCHV1, pMT1 and pMK2 for use in constructing pMTSHV1 or pMKSHV1 as hirudin HV1 secretion plasmids used in the present invention, and of plasmid pUCHV3 for use in constructing plasmid pMTSHV1C3, are provided in the following reference examples.

[Reference example 1]

Preparation of plasmid pUCHV1 and plasmid pUCHV3

10 μg of commercially available plasmid pUC18 was digested with 30 units of EcoRI and 30 units of HindIII at 37 °C for 2 hours. The vector moiety was then separated by agarose gel electrophoresis followed by extraction. Proteins were removed by phenol extraction; DNA was precipitated with cold ethanol and dissolved in 50 μl of TE buffer solution (10mM Tris-HCl, pH8.0, 1mM EDTA). To this amount of solution which should contain 50 ng of DNA, 10 μl of 66mM Tris-HCl, pH7.5, 5mM $MgCl_2$, 5mM DTT, 1mM ATP, and 300 units of T4 DNA ligase containing double stranded HV1 or HV3 DNA was added, followed by reaction overnight at 16 °C to generate plasmid pUCHV1 or pUCHV3, in which the HV1 gene or HV3 gene was inserted, respectively, between the EcoRI and HindIII sites of plasmid pUC18.

[Reference example 2]

Preparation of plasmid pMK2 and plasmid pMT1

(a) Preparation of plasmid pMK2

A fragment containing the tac promoter which was obtained by cleaving commercially available plasmid pKK223-3 (Pharmacia) with the restriction enzymes PvuI and NruI, a fragment containing a replication origin (ori) obtained by cleaving commercially available pUC18 with restriction enzymes PvuI and PvuII, and a fragment containing the ampicillin resistance gene were ligated using T4 DNA ligase. The thus obtained fragment was introduced into E. coli JM109, followed by culturing, screening for ampicillin resistance, and a vector having both the tac promoter and the replication origin (ori) of pUC18 was obtained, and designated as pMK2.

(b) Preparation of plasmid pMT1

10 μg of plasmid pMK2 was digested with 30 units of EcoRI and Eco47III, removing the fragment containing the tac promoter, and then the fragment containing the replication origin (ori) was recovered by agarose gel electrophoresis. The DNA fragment containing the trp promoter was synthesized by a DNA synthesizer. This fragment was ligated with the aforesaid fragment generated by digesting pMK2 with EcoRI and Eco47III, using T4 DNA ligase at 16 °C overnight. The ligation products were used to transform E. coli JM109 to prepare a vector having both the trp promoter and the replication origin (ori) of plasmid pMK2. The DNA sequence of this plasmid was confirmed by the method of Sanger et al., and designated as pMT1.

The present invention is shown in detail hereinafter via the following Example.

[Example 1]

(1) Construction of hirudin HV1 secretion plasmid pMTSHV1

Plasmid pMTSHV1 was constructed according to the procedure shown in Fig. 3. Four oligonucleotides indicated in Fig. 2 were synthesized to construct a DNA fragment corresponding to the signal peptide of alkaline phosphatase (phoA) of E. coli and the DNA fragment encoding $Val^1$-$Val^2$ of the N-terminal amino acid of hirudin HV1. After deprotection, each oligonucleotide was purified by 10% polyacrylamide gel electrophoresis.

After 500 pmol of each of the two oligonucleotides S2 and S4 were phosphorylated, 20 pmol each of the 4 oligonucleotides were mixed, annealed and then treated at 16 °C overnight in 20 μl solution containing T4 DNA ligase. After removal of protein using phenol and chloroform, and precipitation by cold ethanol, the desired double stranded DNA fragment was obtained. 1/10 the amount of the obtained fragment and 100 ng of plasmid pUCHV1 (Japanese Laid Open Patent Publication 3-164184 (1991)) cleaved with restriction enzymes EcoRI and AccI were reacted with T4 DNA ligase at 16 °C overnight. Hybrid plasmid pSHV1, containing the fusion gene in which the DNA sequence encoding hirudin HV1 is ligated immediately after the sequence encoding the phoA signal peptide, was obtained by transforming E. coli JM109. The DNA sequence of this plasmid pSHV1 was confirmed by the method of Sanger et al.

10 µg of pSHV1 was digested with restriction enzymes EcoRI (30 units) and HindIII (30 units), and the 276bp fused gene fragment was purified by agarose gel electrophoresis.

100 ng of the obtained DNA fragment and 100 ng of DNA prepared by digesting E. coli expression vector pMT1 (Japanese Laid Open Patent Publication 3-76579 (1991)) with restriction enzymes EcoRI and HindIII, followed by purifying the DNA fragments by agarose gel electrophoresis, were ligated using T4 DNA ligase at 16°C overnight. The resulting reaction mixture was used to transform E. coli JM109 to obtain the hirudin HV1 secretion plasmid pMTSHV1, in which the fused gene encoding the phoA signal peptide and hirudin HV1 is follows trp promoter region. The DNA sequence of pMTSHV1 was confirmed by the method of Sanger et al.

(2) Construction of the hirudin HV1 secreting plasmid pMKSHV1

Plasmid pMKSHV1 was constructed according to the method shown in Fig. 4.

The aforesaid fused DNA encoding the phoA signal peptide and hirudin HV1, and the DNA fragment obtained by digesting E. coli expression plasmid pMK2 (Japanese Laid Open Patent Publication 3-76579 (1991)) with restriction enzymes EcoRI and HindIII, were ligated using T4 DNA ligase; the ligation products were used to transform E. coli JM109, and the hirudin HV1 secreting plasmid pMKSHV1 having the fused gene inserted downstream of the tac promoter was obtained. The DNA sequence of plasmid pMKSHV1 was confirmed by the method of Sanger et al.

(3) Secretion production of hirudin HV1 by pMTSHV1

E. coli JM109 transformed with plasmid pMTSHV1 or pMKSHV1, constructed in (1) and (2) above, was cultured in 2 x YT medium (16g/l bactotryptone, 10g/l bacto-yeast extract and 5g/l NaCl) containing 100 µg/ml of ampicillin. After culturing at 37°C for 24 hours, 1 ml of culture medium was collected.

Precipitated cells of each sample were suspended in 1ml of 50mM Tris-HCl (pH7.5) containing 25% sucrose and 1mM EDTA, followed by incubation at room temperature for 10 minutes. After collecting the cells by centrifugation at 6,000 x g for 10 minutes, the cells were suspended in 1ml of cold water to release the substances in the periplasmic space by osmotic shock. Cells were removed from the periplasmic fraction by centrifugation at 6,000 x g for 10 minutes. The amount of secreted and periplasmically-accumulated hirudin was determined by measuring the anti-thrombin activity in the supernatant. The antithrombin activity is based on quantitative measurement of the color intensity generated by thrombin's hydrolytic activity on the synthetic chromogenic substrate chromozymeTH (Tocylglycilprolylarginine-4-nitroanilideacetate, Boeringer-Mannheim) and hirudin's inhibitory activity against thrombin, which will repress color genèration.

This reaction was carried out as follows. In the 1ml of said reaction volume, 0.36 NIH U of human-thrombin (Sigma) were added to buffer containing 100mM Tris-HCl (pH8.5), 150mM NaCl and 0.1% polyethyleneglycol-6000, followed by addition of standard hirudin or unknown sample, and followed by pre-incubation at 37°C for 3 minutes.

Both the substrate and chromozymeTH were added to a final concentration of 200 µM to measure the release of p-nitroanilide by the increase of absorbance at 405nm of the solution per 1 minute, and the anti-thrombin activity (ATU) was calculated.

As a result, the strain harboring plasmid pMTSHV1 exhibited 450 ATU of anti-thrombin activity per 1ml of the culture medium. The strain harboring plasmid pMKSHV1 exhibited 360 ATU of anti-thrombin activity per 1ml of the culture medium. Results of further studies on the production of hirudin with various E. coli strains such as JM101, JM103, JM109, TG1, HB101, JA221, IFO3301, C600, RR1 and DH5 in which plasmid pMTSHV1 was introduced by the transformation method of Hanahan et al. are shown in Table 1.

When RR1 was used as host, about 2000ATU/ml of HV1 was produced extracellularly.

Table 1

| Bacterial species | A660nm | Activity (ATU/ml) | Activity of produced HV1 (ATU/ml/A660nm) |
|---|---|---|---|
| JM101 | 4.12 | 256.4 | 62.2 |
| JM103 | 4.12 | 306.7 | 74.4 |
| JM109 | 3.06 | 450.5 | 147.2 |
| TG1 | 5.77 | 185.2 | 32.1 |
| HB101 | 3.76 | 99.0 | 26.3 |
| JA221 | 5.72 | 413.2 | 72.2 |
| IFO3301 | 2.61 | 148.1 | 56.8 |
| C600 | 4.02 | 526.3 | 130.9 |
| RR1 | 7.23 | 2000.0 | 276.6 |
| DH5 | 7.00 | 10.6 | 1.5 |

(4) Secretion of hirudin HV1 into the culture medium by transformed E. coli JM109/pMTSHV1

When E. coli strain JM109 was transformed with plasmid pMTSHV1 (E. coli JM109/pMTSHV1) (FERM BP-3266) and cultured in 2 L of 2 x YT culture medium containing 2% glucose in a 5 L fermenter with agitation and aeration at 37°C for 24 hours, about 5300 ATU per 1ml of hirudin HV1 were produced extracellularly.

(5) Purification of hirudin HV1 from culture media

After fermentation, 1.5 L of culture medium were collected and centrifuged at 6,000 x g for 10 minutes to separate supernatant from cellular debris. Since the salt concentration of the supernatant was 1.3% when it was measured with a salt meter, the supernatant was diluted 4-fold with 10mM potassium phosphate buffer (pH7.0) and filtered through a 3.2μm filter (Pole Co. Ltd). The filtrate was loaded on a QAE-toyopearl column (4.4 x 7cm) equilibrated with 10mM potassium phosphate buffer (pH7.0). After loading the sample, the column was equilibrated in buffer, followed by stepwise elution of hirudin HV1 with 0.2M NaCl. The eluted solution was concentrated using an Amicon Diaflow membrane (YM5), followed by gel filtration on Sephacryl S-100HR pre-equilibrated with 10mM potassium phosphate buffer (pH7.0) for desalting.

The active fractions were loaded on a column of DEAE-toyopearl (4.4 x 40 cm) equilibrated with 10mM potassium phosphate buffer (pH7.0), washed thoroughly, and then eluted with a linear gradient created between 3 L of the equilibration buffer and 3 L of 0.3M NaCl in equilibration buffer. Final purification was carried out on a C4 reverse phase HPLC column with Delta-prep 3000, which is a Waters' product. The purified hirudin HV1 was obtained by eluting it from the column using a linear gradient of 15 to 30% (v/v) acetonitrile containing 0.065% (v/v) trifluoro-acetate.

The degree of purification at each step is shown in Table 2.

Table 2

| Purification step | Total protein (mg) | Total activity (ATU) | relative activity (ATU/mg) | recovery (%) |
|---|---|---|---|---|
| culture medium | 13680 | 6030297 | 441 | 100 |
| QAE- toyopearl | 1324 | 6132812 | 4630 | 101.7 |
| S-100HR | 872 | 6162156 | 7066 | 102.2 |
| DEAE-toyopearl | 821 | 6080019 | 7407 | 100.8 |
| C4RP-HPLC | 570 | 4675211 | 8202 | 77.5 |

The amino acid composition of the thus obtained hirudin HV1 exhibited a value consistent with that of natural hirudin HV1 as shown in Table 3. The N-terminal amino acid sequence of purified hirudin HV1 started with Val-Val-Tyr as shown in Table 4, indicating that correct cleavage of the phoA signal peptide had occurred. The anti-thrombin activity was 8202ATU/mg.

Table 3

| | HV1 | HV1C3 |
|---|---|---|
| Asx | 9.00 | 9.95 |
| Thr | 3.86 | 3.84 |
| Ser | 3.70 | 3.67 |
| Glx | 13.78 | 12.65 |
| Gly | 8.89 | 8.86 |
| Ala | 0.00 | 1.06 |
| Cys | 5.40 | 5.40 |
| Val | 2.94 | 2.92 |
| Met | 0.00 | 0.00 |
| Ile | 1.89 | 1.86 |
| Leu | 4.04 | 3.01 |
| Tyr | 2.06 | 2.06 |
| Phe | 1.00 | 1.00 |
| His | 1.13 | 1.11 |
| Lys | 3.02 | 3.02 |
| Arg | 0.00 | 0.00 |
| Pro | 3.05 | 4.09 |

Table 4

| Cycle number | Amino acid | Yield (pmol) |
|---|---|---|
| 1 | Val | 310 |
| 2 | Val | 490 |
| 3 | Tyr | 189 |
| 4 | Thr | 138 |
| 5 | Asp | 42 |
| 6 | Cys | - |
| 7 | Thr | 44 |
| 8 | Glu | 99 |
| 9 | Ser | 436 |
| 10 | Gly | 205 |
| 11 | Gln | 131 |
| 12 | Asn | 66 |
| 13 | Leu | 148 |
| 14 | Cys | - |
| 15 | Leu | 174 |

(6) Preparation of chimeric hirudin HV1C3 secretion plasmid pMTSHV1C3

Plasmid pMTSHV1C3 was constructed according to the procedure described in Fig. 5. To substitute after the 52nd amino acid of hirudin HV1 with the sequence of HV3, the HV1 secretion plasmid pMTSHV1 (10μg) was digested with restriction enzymes Kpn I (30 units) and Hind III (30 units), followed by agarose gel electrophoresis to purify a DNA fragment of 2.8 Kbp.

Similarly, 10 μg of plasmid pUCHV3 (Japanese Laid Open Patent Publication 3-164184 (1991)) was also digested with Kpn I and Hind III, and an 80 bp DNA fragment encoding the C-terminal amino acid sequence of HV3 was purified.

100 ng of both DNA fragments were reacted with T4 DNA ligase at 16 °C overnight, and the resulting reaction mixture was used to transform E. coli JM109 to obtain chimeric hirudin HV1C3 secretion plasmid pMTSHV1C3. The DNA sequence of plasmid pMTSHV1C3 was confirmed by the method of Sanger et al.

(7) Production of chimeric hirudin HV1C3 by secretion using hirudin secretion plasmid pMTSHV1C3

E. coli RR1 (FERM BP-3130) transformed with plasmid pMTSHV1C3 which was constructed in (6) above was cultured in 2 x YT medium containing 100 μg/ml of ampicillin. After culturing at 37 • C for 24 hours, 1ml of culture medium was collected, and osmotic shock was applied in order to measure the anti-thrombin activity of the periplasmic fraction.

As a result, 3060 ATU of anti-thrombin activity per 1ml of culture medium was detected.

(8) Secretion of chimeric hirudin HV1C3 into the fermentation or culture medium by transformed strain E. coli JM109/pMTSH1C3

When E. coli JM109 (FERM BP-3104) transformed with plasmid pMTSHV1C3 was cultured in 2 L of 2 x YT culture medium containing 2% glucose in a 5L fermenter with agitation and aeration at 37 • C for 24 hours, a total anti-thrombin activity of 6050 ATU/ml, 350ATU/ml in the periplasm, and 5700ATU/ml in the culture medium, was detected.

(9) Purification of chimeric hirudin HV1C3

Hirudin HV1C3 was purified from the culture medium obtained in (8) above according to the method in (5) above. When the amino acid sequence of purified chimeric hirudin HV1C3 was determined, the C-terminal amino acid sequence was confirmed to be different from HV1, as designed, as shown in Fig.1. The specific activity was 11,250 ATU/mg. The HPLC profiles of purified HV1 and HV1C3 are shown in Fig. 6. The experiment was carried out with a VYDAC C4 (0.46 x 25cm) column using a linear gradient of acetonitrile from 15 to 30% at a flow rate of 1 ml/min for 30 minutes.

[Example 2]

Inhibitory action of hirudin against thrombin induced death

Thrombin (10 NIH unit / 10g) was administered intravenously to male mice (20 - 25g), without anesthesia, and the anti-thrombin activity of the tested compound was evaluated by observing the disappearance of roll over reflection and death as indexes. All the tested compounds were dissolved in saline, and 0.05 ml/10g were administered intravenously 5 min prior to the thrombin injection. The results are shown in Table 5.

Table 5

| Inhibitory action of hirudin against thrombin-induced death | | |
|---|---|---|
| Tested compound | Amount administered (μg/10g weight) | Score* |
| Thrombin + Saline (10 NIH unit/10g weight) | | 1.7 |
| Thrombin + HV1 (10 NIH unit/10g weight) | 100<br>200<br>500 | 1.3<br>0.6<br>0.4 |
| Thrombin + HV1C3 (10 NIH unit/10g weight) | 20<br>50<br>100 | 1.2<br>0.6<br>0.4 |

*Score
score 0: no disappearance of roll over reflection (apparently normal)
score 1: disappearance of roll over reflection, but no death within 20 minutes
score 2: death within 20 minutes

As is clear from the table, in the thrombin induced death reaction *in vivo*, chimeric hirudin (HV1C3) of the present invention exhibited about 4 - 5 times greater inhibitory activity compared with hirudin HV1.

[Example 3]

Prolongation of bleeding time

Samples were injected into male mice (20 - 25g) under anesthesia using sodium pentobarbital (40 mg/kg i.p.) via their tail vein. A puncture wound was made by inserting a 21G needle (outer diameter 0.85 mm) into the other side of the tail vein 5 minutes after the administration of the test compounds, and the bleeding time of the wound was measured. A filter paper was put on the wound, with changes every 15 seconds. Bleeding time is defined as the time required until no red spot is observed on the filter paper. The results are shown in Table 6.

Table 6

| Prolongation of bleeding time | | |
|---|---|---|
| Tested compound | Amount administered (μg/10g weight) | Bleeding time (sec) |
| Saline | | 148.5 ± 14.6 |
| HV1 | 20 | 223.7 ± 15.6 |
| | 50 | 376.7 ± 20.2 |
| | 100 | 501.7 ± 47.1 |
| HV1C3 | 50 | 264.0 ± 17.6 |
| | 100 | 291.0 ± 30.2 |
| | 200 | 369.0 ± 34.9 |

In general, prolongation of bleeding time is one of the side effects of anti-coagulants. Here, the chimeric hirudin (HV1C3) of the present invention was clearly confirmed to cause less bleeding than hirudin HV1.

[Example 4]

Preparation having chimeric hirudin HV1C3 as an ingredient

The purified chimeric hirudin HV1C3 of the Example 1-(9) was desalted using Sephadex G25 (Pharmacia), followed by filtering through a 0.22 μm filter under sterile conditions. The solution was dispensed into vials and lyophilized. The thus obtained lyophilized powder of chimeric hirudin HV1C3 can be dissolved in saline and used as an injectable drug.

[Example 5]

Production of hirudin HV3

(1) Construction of a hirudin HV3 secretion plasmid

(i) As shown in Fig 9, the replication origin (ori) obtained by digesting the plasmid pUCHV3 prepared in reference example 1 with restriction enzymes EcoRI and AccI, a vector fragment including the ampicillin resistance gene, and a synthetic gene encoding the signal sequence of alkaline phosphatase (phoA), the DNA sequence of which is shown in Fig. 10, were ligated using T4 DNA ligase. As a result, plasmid pSHV3 was obtained, in which the DNA sequence encoding the signal peptide of the alkaline phosphatase gene (phoA) was joined upstream from the DNA sequence encoding HV3. The plasmid was introduced into E. coli JM109, followed by culturing and screening for ampicillin resistance.
(ii) Next, plasmid pSHV3 was cleaved with restriction enzymes EcoRI and HindIII to obtain a 275bp gene fragment having a DNA sequence encoding the signal peptide of the alkaline phosphatase gene (phoA) joined upstream from the DNA sequence encoding HV3.
(iii) On the other hand, plasmid pMT1 was prepared by the method described in reference example 2. This plasmid comprises a DNA sequence including the replication origin (ori) of plasmid pUC18 and the trp promoter as described in reference example 2. The vector fragment was obtained by cleaving

plasmid pMT1 with the restriction enzymes EcoRI and HindIII.

(iv) The aforesaid 275bp fragment and the vector fragment were ligated using T4 DNA ligase, introduced into E. coli JM109, which was cultured, and screened for ampicillin resistance to obtain plasmid pMTSHV3, the 2.87kb HV3 expression vector which comprises a DNA sequence including the replication origin (ori) of plasmid pUC, the DNA sequence of the trp promoter, a DNA sequence encoding the signal peptide of the alkaline phosphatase gene (phoA), and the DNA sequence encoding HV3.

(2) Secretion production of hirudin HV3 by the hirudin HV3 secretion plasmid

E. coli RR1 (E. coli RR1/pMTSHV3) (FERM BP-3267), transformed with plasmid pMTSHV3 which was constructed by the above described method, was cultured in 2 L of 2 x YT medium containing 100 μg/ml ampicillin and 2% glucose. Culturing was carried out in 2 L of medium in a 5 L jar at 37 • C for 24 hours. As a result, 6073 ATU of anti-thrombin activity per 1ml of culture medium were detected.

(3) Purification of hirudin HV3 from culture media

Hirudin HV3 was obtained from the culture medium by the method described in Example 1-(5). Specifically, after the culture medium was centrifuged to separate the supernatant from the cells, the supernatant was diluted 4-fold with 10mM potassium phosphate buffer (pH7.0) and filtered. The obtained filtrate was loaded on a column (4.4 x 13cm) of QAE-toyopearl as described in example (5), followed by gel filtration on a Sephacryl S-100HR column.

The active fraction was loaded on a column of DEAE toyopearl (4.4 x 40cm) equilibrated with equilibration buffer and then eluted with a linear gradient from 0 to 0.3M NaCl in equilibration buffer. Finally, hirudin HV3 was purified by C4 reverse HPLC using a Vydac C4 column (4.7 x 30cm) eluted with a linear gradient (1 %/min, 30 min or longer) of acetonitrile from 15 to 30% (v/v). The profile is shown in Fig. 11.

The degree of purification of each step is shown in Table 7.

Table 7

| Purification step | Total protein (mg) | Total activity (ATU) | Relative activity (ATU/mg) | Recovery (%) |
|---|---|---|---|---|
| culture medium | 15600 | 11034400 | 707 | 100 |
| QAE- toyopearl | 2111 | 9227264 | 4371 | 83.6 |
| S-100HR | 1453 | 9268963 | 6381 | 84.0 |
| DEAE-toyopearl | 1327 | 8597892 | 6478 | 77.9 |
| C4-HPLC | 795 | 6486480 | 8139 | 58.8 |

When the amino acid composition and N-terminal amino acid sequence of the purified product were analyzed, the amino acid composition exhibited a value consistent with the theoretical value of hirudin HV3 as shown in Table 8. As shown in Table 9, the sequence up to the 15th amino acid of the N-terminal amino acid sequence was identical with that of hirudin HV3. These results evidence the correct processing of the signal peptide, and the product was confirmed to be hirudin HV3.

Table 8

| | HV3 | Theoretical |
|---|---|---|
| Asx | 9.76 | 10 |
| Thr | 4.74 | 5 |
| Ser | 3.68 | 4 |
| Glx | 11.55 | 11 |
| Gly | 8.77 | 9 |
| Ala | 1.07 | 1 |
| Cys | 5.22 | 6 |
| Val | 2.03 | 2 |
| Met | 0.00 | 0 |
| Ile | 2.97 | 3 |
| Leu | 3.08 | 3 |
| Tyr | 2.04 | 2 |
| Phe | 1.00 | 1 |
| His | 1.22 | 1 |
| Lys | 4.03 | 4 |
| Arg | 0.00 | 0 |
| Pro | 4.06 | 4 |
| Total | | 66 |

Table 9

| Cycle number | Amino acid | Yield (pmol) |
|---|---|---|
| 1 | Ile | 469 |
| 2 | Thr | 108 |
| 3 | Tyr | 106 |
| 4 | Thr | 102 |
| 5 | Asp | 122 |
| 6 | Cys | - |
| 7 | Thr | 100 |
| 8 | Glu | 76 |
| 9 | Ser | 32 |
| 10 | Gly | 172 |
| 11 | Gln | 145 |
| 12 | Asn | 93 |
| 13 | Leu | 211 |
| 14 | Cys | - |
| 15 | Leu | 189 |

## Industrial Applicability

The present invention provides a new hirudin analog. The hirudin analog of the present invention is useful as an anti-coagulant having strong anti-thrombin activity and a tendency to cause bleeding.

Also, the present invention provides secretion vectors for secretion of foreign proteins including the aforesaid hirudin analog, transformed microorganisms, and methods of manufacturing foreign proteins using said transformed microorganisms. Using the method described in the present invention, foreign proteins can be produced extracellularly in high yield, which is industrially advantageous for obtaining foreign proteins.

Deposits of microorganisms:

(1) E. coli JM109/pMTSHV1C3
(Entry E. coli JM109/pMTPHOHV1C3 was amended)
Deposit Organization:
Fermentation Research Institute,
Agency of Industrial Science and Technology, Ministry of International Trade and Industry
Address:
1-1-3, Tsukuba-shi Higashi, Ibaraki-ken, Japan
Date of Deposit:
September 18, 1990
Deposit Number:
FERM BP-3104

(2) E. coli RR1/pMTSHV1C3
Deposit Organization:
Fermentation Research Institute,
Agency of Industrial Science and Technology, Ministry of International Trade and Industry
Address:
1-1-3, Tsukuba-shi Higashi, Ibaraki-ken, Japan
Date of Deposit:
October 11, 1990
Deposit Number:
FERM BP-3130

(3) E. coli JM109/pMTSHV1
Deposit Organization:
Fermentation Research Institute,
Agency of Industrial Science and Technology, Ministry of International Trade and Industry
Address:
1-1-3, Tsukuba-shi Higashi, Ibaraki-ken, Japan
Date of Deposit:
February 6, 1991
Deposit Number:
FERM BP-3266

(4) E. coli RR1/pMTSHV3
Deposit Organization:
Fermentation Research Institute,
Agency of Industrial Science and Technology, Ministry of International Trade and Industry
Address:
1-1-3, Tsukuba-shi Higashi, Ibaraki-ken, Japan
Date of Deposit:
February 6, 1991
Deposit Number:
FERM BP-3267

## Claims

1. A secretion vector for foreign protein production, comprising a DNA sequence comprising a replication origin (ori) of a pUC plasmid, a tac or a trp promoter, a DNA sequence encoding signal peptide, and a DNA sequence encoding a foreign protein, all linked in an operable manner.

2. A secretion vector according to claim 1, wherein said DNA sequence encoding said signal peptide is derived from alkaline phosphatase.

3. A. secretion vector according to claims 1 or 2, wherein said DNA sequence of said replication origin (ori) of said pUC plasmid is obtained by digesting plasmid pUC18 with the restriction enzymes PvuI and PvuII.

4. A secretion vector according to any one of claims 1 to 3, wherein said foreign protein is hirudin HV1.

5. A secretion vector according to any one of claims 1 to 3, wherein said foreign protein is hirudin HV3.

6. A secretion vector according to any one of claims 1 to 3, wherein said foreign protein is hirudin analog HV1C3 having amino acid sequence of Formula-1, below:

```
Val Val Tyr Thr Asp Cys Thr Glu Ser Gly
1               5                  10
Gln Asn Leu Cys Leu Cys Glu Gly Ser Asn
            15                  20
Val Cys Gly Gln Gly Asn Lys Cys Ile Leu
            25                  30
Gly Ser Asp Gly Glu Lys Asn Gln Cys Val
            35                  40
Thr Gly Glu Gly Thr Pro Lys Pro Gln Ser
            45                  50
His Asn Gln Gly Asp Phe Glu Pro Ile Pro
            55                  60
Glu Asp Ala Tyr Asp Glu
            65
```

(Formula-1)

7. Transformed microorganisms wherein E. coli was transformed with a secretion vector according to any one of claims 1 to 6.

8. A method of manufacturing hirudin HV1, HV3 or hirudin analog HV1C3 of Formula-1, comprising culturing the transformed microorganism of claim 7 and recovering the product from the culture medium.

Fig. 1

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HV1 | Val | Val | Tyr | Thr | Asp | Cys | Thr | Glu | Ser | Gly | Gln | Asn | Leu | Cys | Leu |
| HV2 | Ile | Thr | Tyr | Thr | Asp | Cys | Thr | Glu | Ser | Gly | Gln | Asn | Leu | Cys | Leu |
| HV3 | Ile | Thr | Tyr | Thr | Asp | Cys | Thr | Glu | Ser | Gly | Gln | Asn | Leu | Cys | Leu |
| HV1C3 | Val | Val | Tyr | Thr | Asp | Cys | Thr | Glu | Ser | Gly | Gln | Asn | Leu | Cys | Leu |

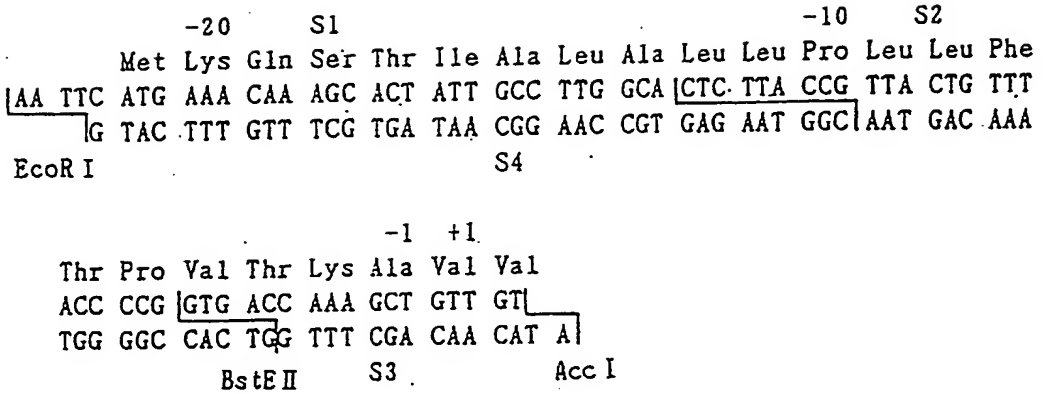
| | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cys | Glu | Gly | Ser | Asn | Val | Cys | Gly | Gln | Gly | Asn | Lys | Cys | Ile | Leu |
| | Cys | Glu | Gly | Ser | Asn | Val | Cys | Gly | Lys | Gly | Asn | Lys | Cys | Ile | Leu |
| | Cys | Glu | Gly | Ser | Asn | Val | Cys | Gly | Lys | Gly | Asn | Lys | Cys | Ile | Leu |
| | Cys | Glu | Gly | Ser | Asn | Val | Cys | Gly | Gln | Gly | Asn | Lys | Cys | Ile | Leu |

| | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gly | Ser | Asp | Gly | Glu | Lys | Asn | Gln | Cys | Val | Thr | Gly | Glu | Gly | Thr |
| | Gly | Ser | Asn | Gly | Lys | Gly | Asn | Gln | Cys | Val | Thr | Gly | Glu | Gly | Thr |
| | Gly | Ser | Gln | Gly | Lys | Asp | Asn | Gln | Cys | Val | Thr | Gly | Glu | Gly | Thr |
| | Gly | Ser | Asp | Gly | Glu | Lys | Asn | Gln | Cys | Val | Thr | Gly | Glu | Gly | Thr |

| | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pro | Lys | Pro | Gln | Ser | His | Asn | Asp | Gly | Asp | Phe | Glu | Glu | Ile | Pro |
| | Pro | Asn | Pro | Glu | Ser | His | Asn | Asn | Gly | Asp | Phe | Glu | Glu | Ile | Pro |
| | Pro | Lys | Pro | Gln | Ser | His | Asn | Gln | Gly | Asp | Phe | Glu | Pro | Ile | Pro |
| | Pro | Lys | Pro | Gln | Ser | His | Asn | Gln | Gly | Asp | Phe | Glu | Pro | Ile | Pro |

| | 61 | 62 | 63 | 64 | 65 | 66 |
|---|---|---|---|---|---|---|
| | Glu | Glu | Tyr | Leu | Gln | |
| | Glu | Glu | Tyr | Leu | Gln | |
| | Glu | Asp | Ala | Tyr | Asp | Glu |
| | Glu | Asp | Ala | Tyr | Asp | Glu |

Fig. 2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S4
-1
+1
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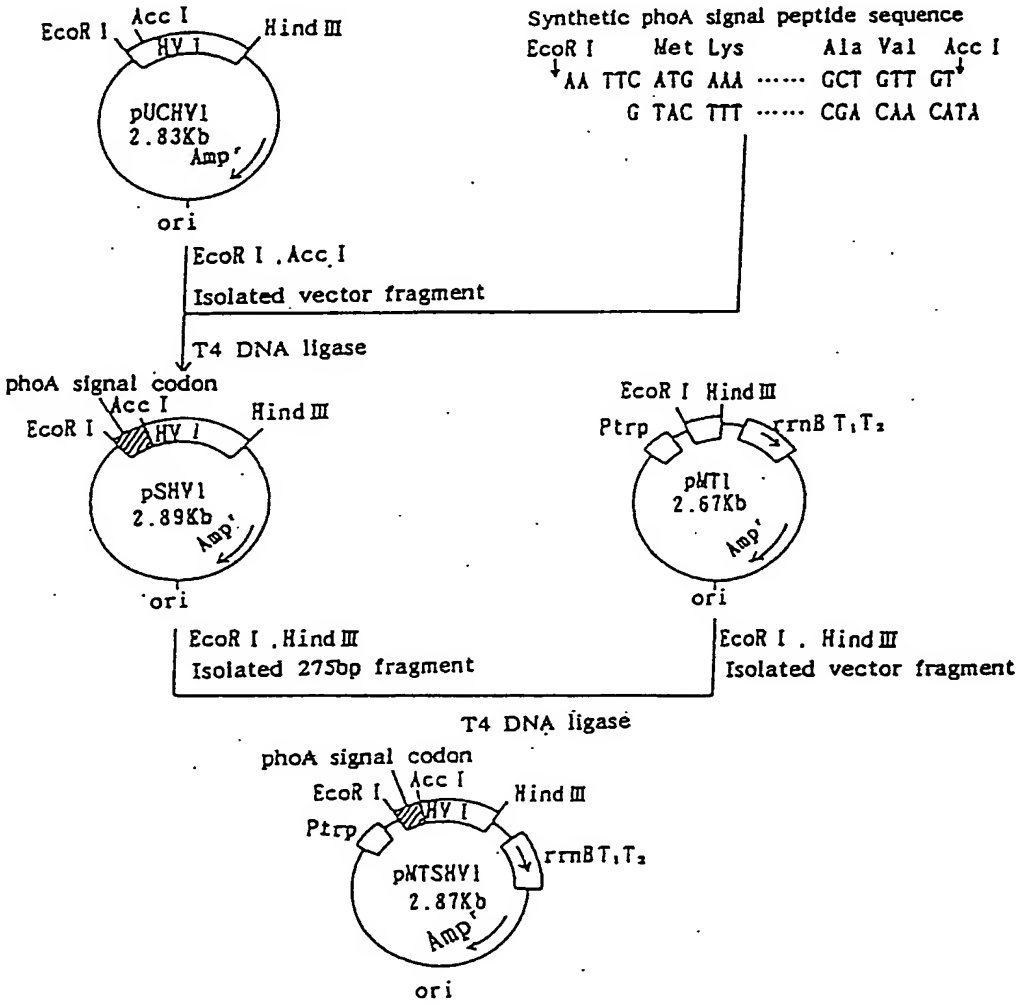
BstE II
S3
Acc I

Fig. 3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Ptrp
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Isolated vector fragment
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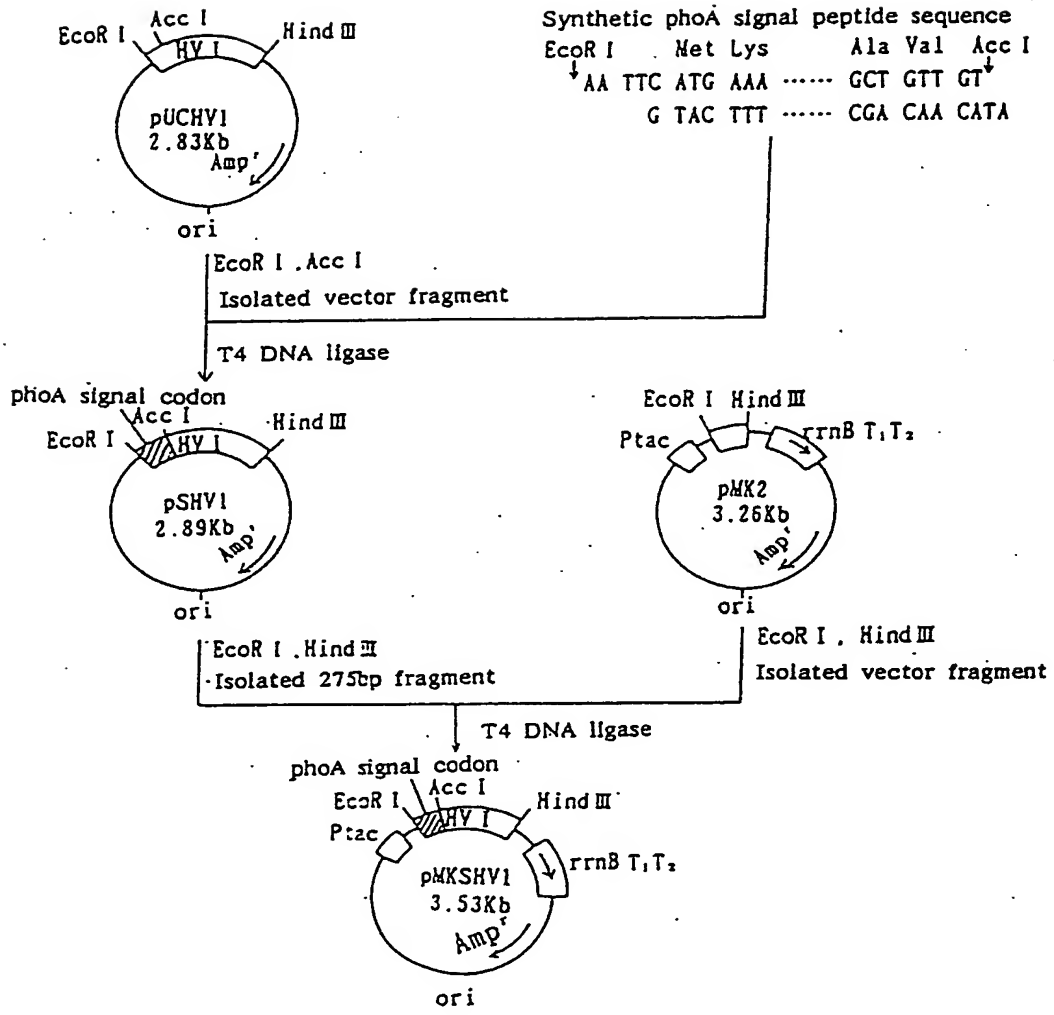
2.87Kb

Fig. 4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pSHV1
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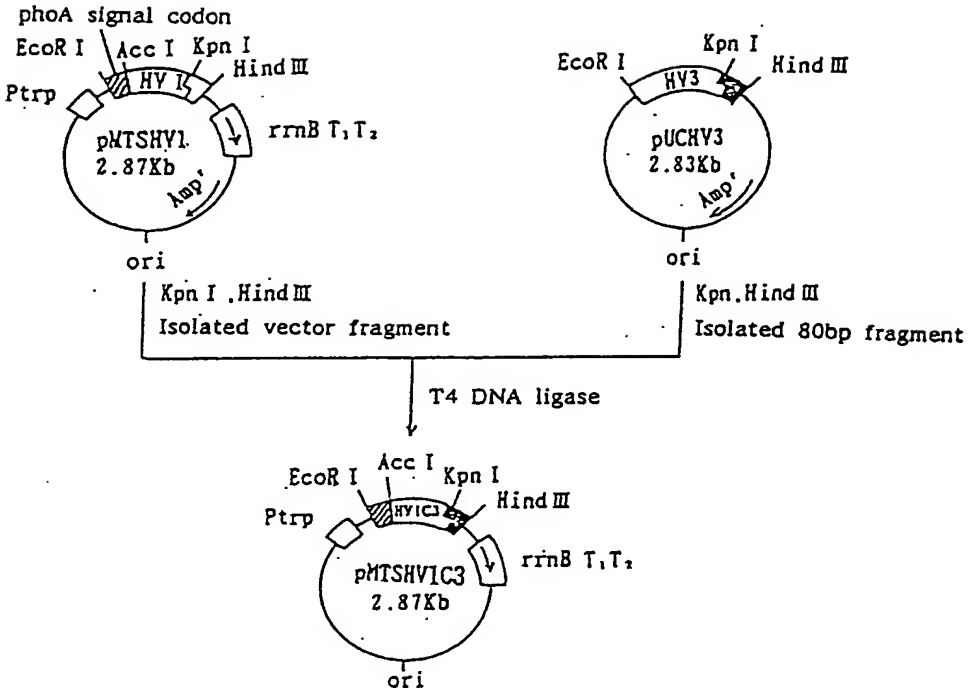
Fig. 5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ori
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ori

Fig. 6

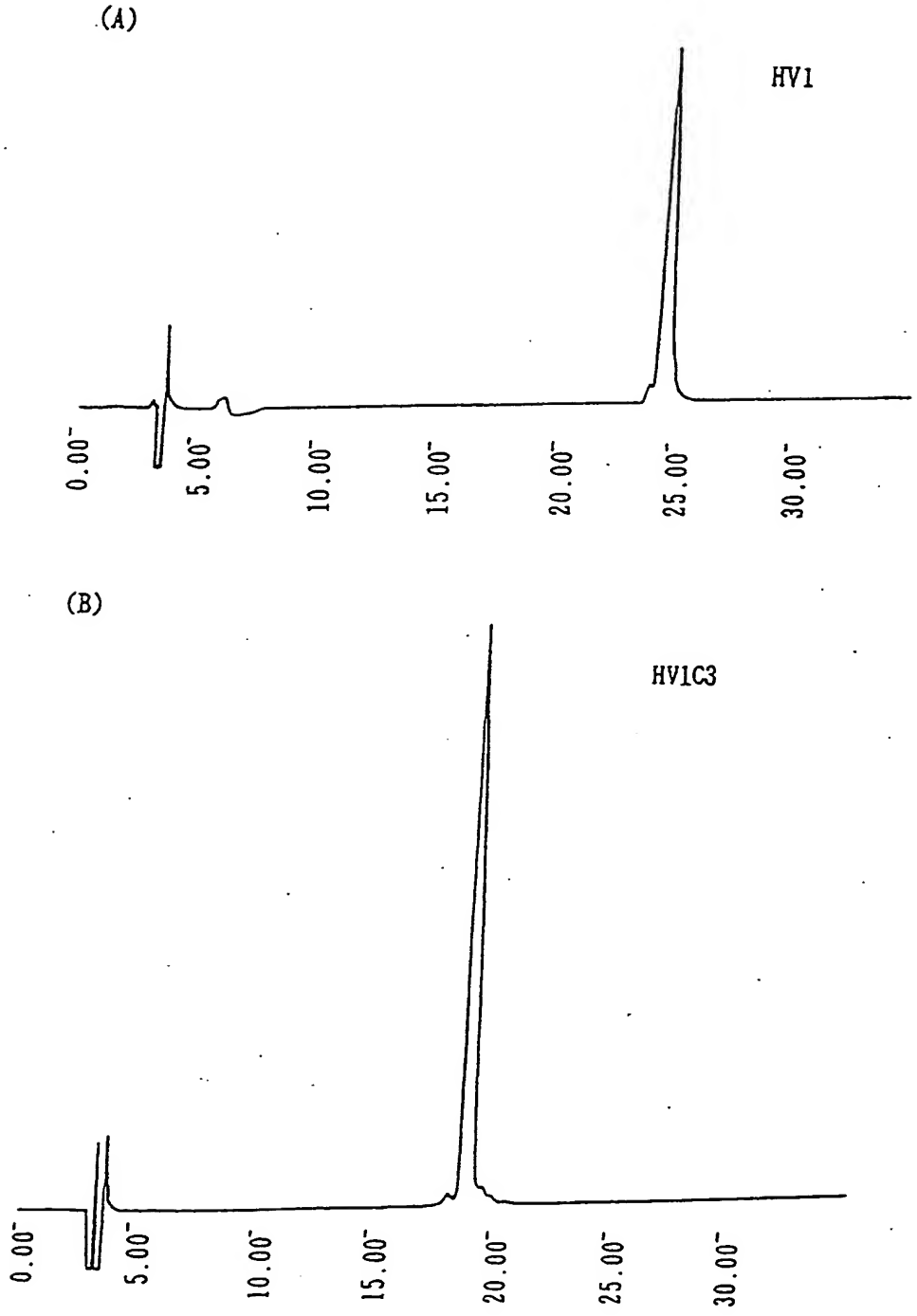
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Fig. 7

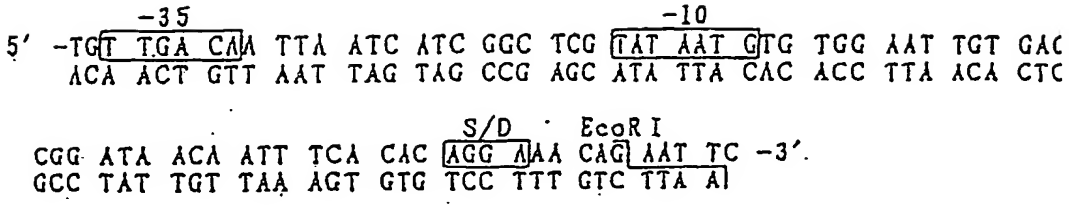
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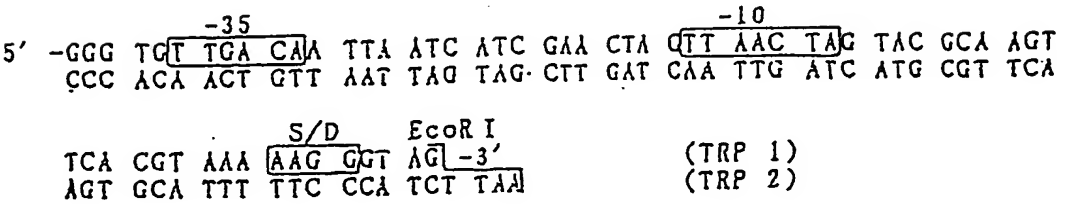
Fig. 8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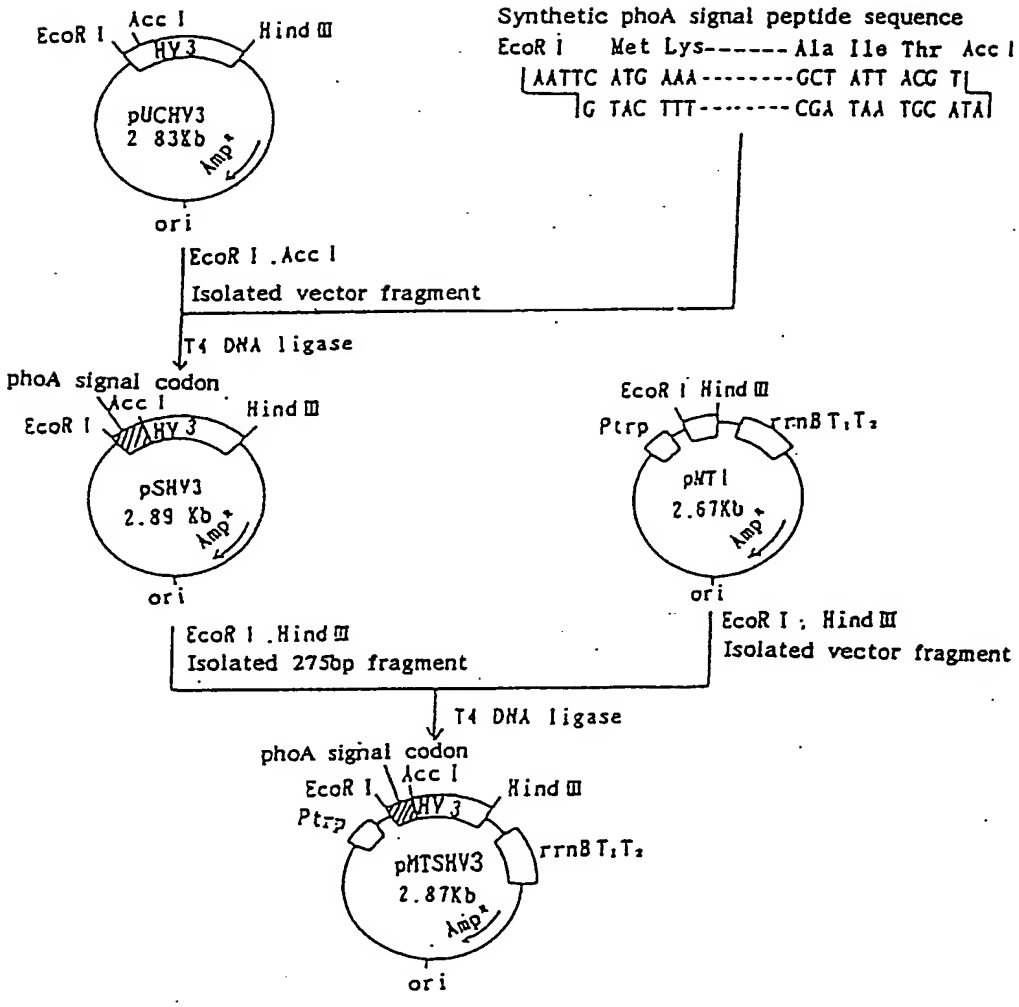
(TRP 2)

Fig. 9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AATTC ATG AAA--------GCT ATT ACG T
G TAC TTT--------CGA TAA TGC ATA
EcoR I .Acc I
Isolated vector fragment
T4 DNA ligase
phoA signal codon
pSHV3
2.89 Kb
Ptrp
rrnB T1T2
pMT1
2.67Kb
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Isolated 275bp fragment
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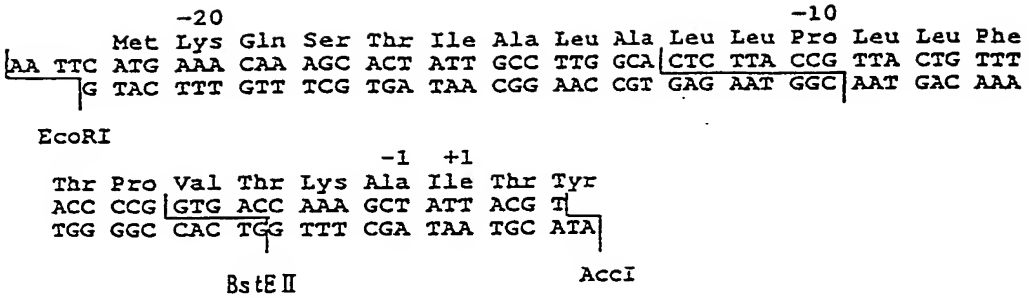
2.87Kb

Fig. 10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G TAC TTT GTT TCG TGA TAA CGG AAC CGT GAG AAT GGC AAT GAC AAA
EcoRI
-1 +1
Thr Pro Val Thr Lys Ala Ile Thr Tyr
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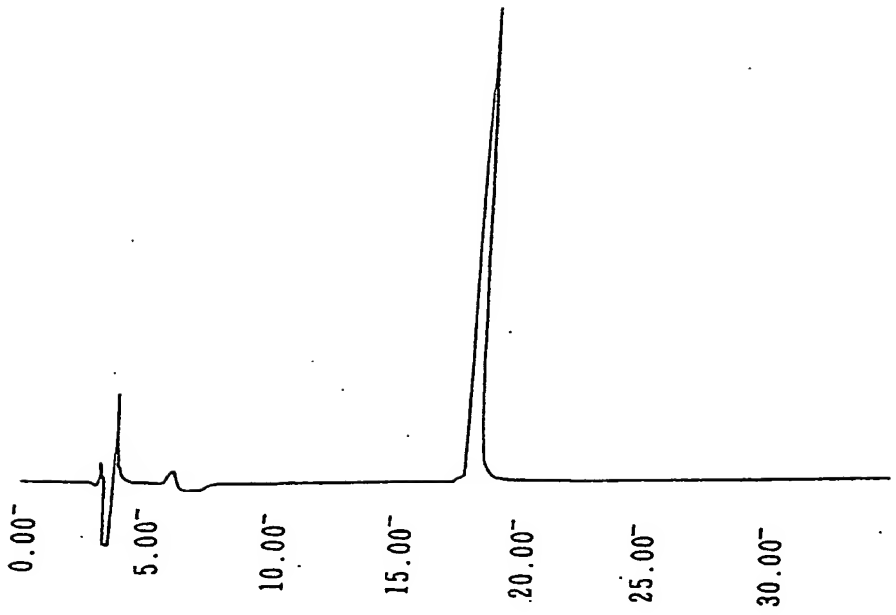
BstEII
AccI

Fig.11



European Patent Office

# EUROPEAN SEARCH REPORT

Application Number
EP 95 20 2092

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.5) |
|---|---|---|---|
| X<br>Y | EP-A-0 352 839 (NIPPON SHINYAKU CO LTD) 31 January 1990<br>* page 4, line 50 - page 5, line 45 *<br>--- | 1,2,7<br>4,5,8 | C12N15/12<br>C12N15/62<br>C12N15/70<br>C12N1/21 |
| X<br>Y | EP-A-0 177 343 (GENENTECH INC) 9 April 1986<br>* the whole document *<br>--- | 1,7<br>4,8 | C07K14/815<br>//(C12N1/21, C12R1:19) |
| D,X<br>Y | FEBS LETTERS, vol. 202, no. 2, June 1986 ELSEVIER, AMSTERDAM, NL, pages 373-377, J. DOTH ET AL. 'Expression, secretion and processing of hirudin in E. coli using the alkaline phosphatase signal sequence'<br>* the whole document *<br>--- | 1,2,4,7, 8<br>5 | |
| X<br>Y | SEMINARS IN THROMBOSIS AND HEMATOSTASIS, vol. 15, no. 3, 1989 THIEME, NEW YORK, US, pages 302-315, P.H. JOHNSON ET AL. 'Biochemistry and genetic engineering of hirudin'<br>* page 304, left column, line 3 - page 305, left column, line 13; figure 1; table 1 *<br>--- | 1,2,4,7, 8<br>5 | TECHNICAL FIELDS SEARCHED (Int.Cl.5)<br>C12N<br>C07K |
| Y | BIOCHEMISTRY, vol. 27, no. 17, 23 August 1988 AM. CHEM. SOC.,WASHINGTON,DC,US, pages 6517-6522, P.J. BRAUN ET AL. 'Use of site-directed mutagenesis to investigate the basis for the specificity of hirudin'<br>* the whole document *<br>--- | 4,8 | |
| | -/-- | | |

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 19 September 1995 | Hornig, H |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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# EUROPEAN SEARCH REPORT

Application Number
EP 95 20 2092

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| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.5) |
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The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
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| THE HAGUE | 19 September 1995 | Hornig, H |

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